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## Effects of DNA and protein size on substrate cleavage by human tyrosyl-DNA phosphodiesterase 1 (TDP1)

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### Abstract

Tyrosyl-DNA phosphodiesterase 1 (TDP1) catalyzes the hydrolysis of phosphodiester linkages between a DNA 3' phosphate and a tyrosine residue as well as a variety of other DNA 3' substituents, and has been implicated in the repair of covalent complexes involving eukaryotic type IB topoisomerases. To better understand the substrate features that are recognized by TDP1, the size of either the DNA or protein component of the substrate was varied. Competition experiments and gel shift analyses comparing a series of substrates with DNA lengths increasing from 6 to 28 nucleotides indicated that, contrary to predictions based on the crystal structure of the protein, the apparent affinity for the substrate increased as the DNA length was increased over the entire range tested. It has previously been found that a substrate containing the full-length native form of human topoisomerase I protein is not cleaved by TDP1. Protein-oligonucleotide complexes containing either a 53 or 108 amino acid long topoisomerase I-derived peptide were efficiently cleaved by TDP1, but like the full length protein, a substrate containing a 333 amino acid topoisomerase I fragment was resistant to cleavage. Consistent with these results, evidence is presented that processing by the proteasome is required for TDP1 cleavage in vivo.

### Keywords

tyrosyl-DNA phosphodiesterase; DNA topoisomerase I; DNA repair; proteasome

### Introduction

Cleavage of the DNA backbone by a topoisomerase is accompanied by the formation of a covalent bond between a tyrosine side chain in the enzyme and the phosphate at the site of cleavage [1]. The type I topoisomerases cleave one of the two DNA strands to form a covalent intermediate that involves a 5'-phosphate for the members of the type IA subfamily and a 3'-phosphate for the members of the type IB subfamily. Human topoisomerase I belongs to the type IB subfamily and provides temporary single-strand breaks to enable the removal of torsional stress associated with DNA replication, transcription, recombination, and likely chromatin remodeling [1-4].

Although topoisomerase I cleavage is normally followed by religation of the DNA, the presence of nicks, strand gaps, mismatched bases, or modified nucleotides in the DNA near

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where the topoisomerase cleaves can cause a failure in religation and trap the topoisomerase on the DNA [5]. In addition, members of the camptothecin family of anticancer drugs specifically intercalate into the DNA at the site of topoisomerase I cleavage and block religation [6-9]. Genetic studies in yeast indicate that multiple pathways exist for the repair of this type of topoisomerase I-mediated DNA damage [10-13]. These studies have implicated tyrosyl-DNA phosphodiesterase (TDP1) [14] and the structure-specific endonucleases Rad1/Rad10 and Mus81/Mms4 in these repair processes. TDP1 is also implicated in the repair of stalled topoisomerase I complexes in human cells [15-18]. The strong preference of TDP1 for removing a tyrosine residue from the ends of single- or double-stranded DNA substrates *in vitro* as opposed to a tyrosine located at a nick on duplex DNA [13, 14, 19, 20] suggests an involvement of the enzyme in the removal of adducts from ends rather than internal sites on DNA. However, TDP1 has also been implicated in the repair of single-strand breaks caused by abortive topoisomerase I-DNA complexes [16] and by ionizing radiation [21].

TDP1 was discovered by virtue of its ability to hydrolyze a tyrosyl O-4 ester linkage to a DNA 3' phosphate [14], but more recently has been shown to also catalyze the hydrolytic removal of a variety of other moieties from the 3' end of DNA. One such adduct is a 3'-phosphoglycolate substituent which is consistent with a role for TDP1 in the repair of damage caused by ionizing radiation [10, 22, 23]. In addition, we previously showed that 3' ends with a free hydroxyl (a nucleoside), an abasic residue, or an artificial biotin adduct are substrates for TDP1 [24]. It has also been reported that yeast TDP1 is capable of hydrolyzing DNA 5' phosphotyrosyl linkages [25], suggesting a possible role for the yeast enzyme in the repair of topoisomerase II covalent complexes, although such a 5' processing activity has not been found for human TDP1 [14, 20]. Notably, a human enzyme with a 5' cleavage activity has recently been reported and referred to as TDP2 (TTRAP) [26].

As with other members of the phospholipase D superfamily [27-29], the reaction catalyzed by TDP1 proceeds through a covalent intermediate in which an active site histidine (His263 in human TDP1) is linked by a phosphoamide bond to the DNA 3' phosphate from the substrate [29, 30]. As an example of the versatility of the enzyme, TDP1 can also hydrolyze this DNA 3' phosphoamide linkage [24, 29, 31, 32], a reaction that likely explains the recessive nature of the neurodegenerative disorder spinocerebellar ataxia with axonal neuropathy (SCAN1) [33] in which a H493R mutation in the *TDP1* gene causes the accumulation of the TDP1-DNA covalent intermediate *in vivo* [17, 18].

By exploiting our observation that vanadate will promote the assembly of a quaternary complex involving TDP1, a short DNA oligonucleotide, and a peptide, we previously used X-ray crystallography to characterize the transition state for the reaction that forms the enzyme-DNA covalent intermediate [31, 34]. These data provide key insights into the mechanism of the reaction and how the protein and DNA moieties of the substrate interact with the enzyme in the immediate vicinity of the active center. Notably, a groove exists that emanates from the active center and appears capable of binding a single strand of DNA approximately 6 nucleotides in length. Paradoxically, this groove is too narrow to accommodate a double-stranded region of DNA. In opposition to this groove is a relatively large concave pocket that accommodates the peptide moiety of the substrate. Attempts to structurally model an intact version of human topoisomerase I into this pocket and maintain the correct configuration for TDP1 cleavage were unsuccessful [31]. This finding would explain the inability of TDP1 to remove an intact topoisomerase I from the 3' end of DNA [24, 35] and is consistent with an earlier suggestion that the topoisomerase I must be modified *in vivo*, most likely by unfolding or proteolysis, before TDP1 cleavage [14].

Here we vary the size of both the DNA and the protein portions of the substrate to elucidate how the substrate interacts with the enzyme at regions more distal from the active center. Specifically, we investigated the effects of varying the DNA length from 6 to 28 nucleotides for a substrate containing a 7 amino acid long topoisomerase I-derived peptide. We also varied the size of the protein moiety of the substrate to gain further insights into the extent of proteolysis required prior to TDP1 cleavage. Finally, we show that the activity of the proteasome is required *in vivo* for TDP1 cleavage of topoisomerase I-DNA covalent complexes.

## Materials and Methods

### Proteins and reagents

The procedures for the purification of topo70, an N-terminal truncated form of human topoisomerase I missing the first 174 amino acids, topo31, and human TDP1 have been previously published [29, 36]. The generation of the following reconstituted forms of topoisomerase I has been described previously: topo56/6.3, topo58/12, and topo31/topo39 [37, 38]. Antibodies against human TDP1 were generated as described [17]. Human scleroderma serum (Scl-70 from Immunovision, Springdale, Arizona) was used in the ICE immunoblot experiments to detect topoisomerase I. Affinity purified rabbit antibodies against human topoisomerase I were used for the whole cell extract immunoblots. Anti-actin antibodies were purchased from Sigma.

### Preparation of TDP1 substrates

The preparation of the 12-mer oligonucleotide with a 3' trypsin-resistant peptide derived from human topoisomerase I (12-pep) has been described previously [29]. Briefly, topo70 was incubated with a partially duplex suicide substrate (CL14N:CP25N) containing a strong topoisomerase I cleavage site two nucleotides from the 3' end of the scissile strand (CL14N: 5' GAAAAAAGAGTTAG 3'). After allowing sufficient time for essentially all of the substrate to become covalently linked to the enzyme, the products were treated with trypsin and the shortened scissile strand was gel-purified and 5' end-labeled with polynucleotide kinase (New England Biolabs) and [ $\gamma$ -<sup>32</sup>P]ATP (NEN Life Sciences Products) prior to use as a substrate for TDP1. The other peptide substrates (6-pep, 19-pep and 28-pep) were similarly prepared using suicide substrates with the appropriate length scissile strands (CL8: 5' AGAGTTAG 3'; CL21: TAGAGGATCTAAAAGACTTAG 3'; CL30: GGTGACTCTAGAGGATCTAAAAGACTTAG 3').

A series of topoisomerase I-derived protein fragments containing a covalently attached 12-mer oligonucleotide were prepared as follows. Topo70 and the reconstituted forms of the enzyme (topo56/6.3, topo58/12, and topo31/39) were incubated with the CL14:CP25N suicide substrate and the respective covalent complexes containing the 12-mer oligonucleotide (12-topo70, 12-topo6.3, 12-topo12, and 12-topo39) were purified by SDS-PAGE (15% gel). The proteins were denatured with guanidine hydrochloride and renatured as described by Hager and Burgess [39]. The suicide substrate contains a second weak cleavage site four nucleotides from the 3' end of the scissile strand that generates a small amount of 10-topo70, 10-topo6.3, 10-topo12, and 10-topo39. After trypsin treatment, these substrates are converted to 10-pep which along with the corresponding TDP1 product, 10-P, are visible in the sequencing gels used for the analysis of the TDP1 reactions.

### Competition TDP1 cleavage reactions

Pairs of labeled peptide substrates (19pep/12-pep, 12-pep/6-pep, 19-pep/6-pep, and 28-pep/19-pep) at a final concentration of 2 nM each were incubated with TDP1 at room temperature (22 °C) for the indicated times in Assay Buffer (10 mM Tris-HCl (pH 7.5), 100

mM KCl, 1 mM EDTA, 1 mM DTT). The enzyme concentration was 23 nM except for the 19-pep/12-pep pair where the enzyme concentration was 4.6 nM. Samples were removed at various time intervals and the reactions quenched by addition of an equal volume of formamide loading buffer as described previously [29]. The products were analyzed by electrophoresis in a 15% sequencing gel, the dried gels were scanned using a PhosphorImager, and the band intensities determined using ImageQuant software (Amersham Biosciences). For purposes of quantitation, the substrates and products resulting from cleavage at the both the primary cleavage site and the weaker secondary cleavage site were summed.

### Gel shift experiments

To prevent cleavage of the peptide substrates during the course of the experiment, the catalytically inactive H263A mutant form of TDP1 [29] was used for the gel shift analyses. The four labeled peptide substrates (6-pep, 12-pep, 19-pep, and 28-pep) (0.15 nM) were incubated at 4°C in Assay Buffer containing 12.5% glycerol (final volume 20  $\mu$ l) with two-fold increasing concentrations of TDP1 (from 23 nM up to 1.46  $\mu$ M) and the samples (16  $\mu$ l portions) were analyzed by electrophoresis in a nondenaturing 5% gel at 4°C as described [40, 41]. The gels were dried, scanned using a PhosphorImager and band intensities determined using ImageQuant software as described above. For the 19-pep and the 28-pep substrates,  $K_d$  values were estimated from the concentration of substrates at which half of the substrate was shifted in mobility [42].

### TDP1 cleavage of the protein-oligonucleotide substrates

For the cleavage assays, the protein-oligonucleotide covalent complexes (12-topo70, 12-topo39, 12-topo12, and 12-topo6.3) were incubated with TDP1 in Assay Buffer at room temperature for 20 min. The reactions were stopped by the addition of SDS (0.6%), followed by precipitation with ethanol and treatment with trypsin [17] to convert all of residual substrate to 12-pep and a small amount of 10-pep before analysis in a 15% sequencing gel.

The religation controls were performed as follows. The religation oligonucleotide pair consisted of CP25N (the noncissile strand of the suicide substrate) which was complementary at its 3' end to the 12-mer attached to the proteins and a 13-mer acceptor oligonucleotide (REL13) which was annealed to the 5' half of CP25N. Religation by the 12-topo70 covalent complex was initiated by the addition of the religation oligonucleotide pair. To enable the covalently bound protein fragments to carry out religation, topo31 was added to 12-topo39, and topo58 was added to 12-topo12 and 12-topo6.3. Religation generated a labeled 25-mer oligonucleotide product that was visualized on the sequencing gel used for the assays. To simplify the analyses, the samples were treated with trypsin as described above to reduce all of the protein-oligonucleotide complexes to 12-pep (and the minor 10-pep species) prior to analysis on a 15% sequencing gel.

### Cell culture and ICE assays

SCAN1 fibroblasts were treated for 1 h with 20  $\mu$ M CPT (Sigma) and the cells lysed immediately with 0.8 % SDS and analyzed by a modified ICE assay [18, 43]. Where indicated, the cells were pretreated for 1 h with 20  $\mu$ M MG132 or for 2h with 40  $\mu$ M lactacystin prior to, and during the CPT treatment. DNA in the extract was lightly sheared by passage through a syringe and the samples diluted approximately 8-fold with 1% N-lauroyl-sarcosine before being loaded onto a 1.5 g/cc CsCl cushion. After centrifugation at 20°C for 20 h at 165,000  $\times$  g (Beckman SW41 Ti rotor), the pellet fraction containing the bulk of the high molecular DNA plus any covalently bound proteins was treated with 300 units of micrococcal nuclease in 20 mM Tris-HCl (pH 8.0), 10 mM NaCl, 2mM CaCl<sub>2</sub> in a final volume of 50  $\mu$ l. The samples were subjected to an immunoblot analysis in which the

membrane was first probed for TDP1 and then topoisomerase I as described [17]. The whole cell extracts were similarly analyzed on immunoblots with probing for TDP1, topoisomerase I, and  $\beta$ -actin.

## Results

### Effect of DNA length on TDP1 cleavage

To compare the effect of DNA length on substrate preference for TDP1, we chose to utilize a form of the substrate where the protein moiety was a short 7 amino acid long trypsin-resistant peptide derived from human topoisomerase I [24, 29]. This choice ensured that the protein moiety would be small enough to be compatible with efficient and unhindered cleavage while at the same time preserving the immediate amino acid context of a known natural substrate of the enzyme. To generate the substrate, human topoisomerase I was incubated with a DNA oligonucleotide suicide substrate until cleavage was complete, followed by treatment with trypsin and gel purification (see Material and Methods). Since the  $K_m$  of human TDP1 is in the micromolar range [44, 45] and we could only prepare sufficient quantities of these substrates to achieve nanomolar concentrations, we were unable to employ standard Michaelis-Menten substrate saturation experiments to directly measure the kinetic parameters for the reaction as a function of DNA length. However, the ratio of the reaction velocities for two competing substrates A and B ( $v_A/v_B$ ) when present at the same concentration in a reaction is equal to the ratio of the specificity constants ( $k_{cat}/K_m$ ) for the substrates as shown by the relationship below [46]. Importantly, this equation holds for all substrate concentrations.

$$\frac{V_A}{V_B} = \frac{(k_{cat}/K_m)_A}{(k_{cat}/K_m)_B}$$

Since this type of analysis does not yield true values for  $k_{cat}/K_m$ , the objective of these experiments was to compare the rates of cleavage for two competing substrates in a reaction as an indicator of the relative specificity constants for the substrates. In this way, we could order the substrates with different DNA lengths according to their relative efficiencies of cleavage. Initially, three DNA-peptide substrates were prepared containing 5'  $^{32}\text{P}$  end-labeled single-stranded oligonucleotides with lengths of 6, 12 and 19 (referred to as 6-pep, 12-pep and 19-pep, respectively) and competed in pair wise combinations for cleavage by human TDP1. For all three substrate pairs tested, the substrates and the corresponding TDP1 products containing 3' phosphates had distinct mobilities in a sequencing gel, enabling us to follow both the disappearance of the substrates and the kinetics of appearance of the products in the competition reactions (Figure 1). As can be seen in the sequencing gel analyses shown in Figure 1(A), the rate of cleavage of 12-pep exceeded that of 6-pep while 19-pep in turn was cleaved faster than 12-pep and not surprisingly, 19-pep was cleaved much faster than 6-pep. These results were quantified by PhosphorImager analysis of three independent experiments for each pair and the rate of disappearance for the 19-pep/12-pep and 12-pep/6-pep pairs is shown graphically in Figs. 1B and 1C. To extend these results to a substrate with yet a longer DNA, we similarly compared 28-pep with 19-pep as substrates and again the longer substrate was cleaved faster (Figure 2).

These results were surprising in view of the crystal structure of TDP1 showing that the most likely binding trajectory for single-stranded DNA follows a narrow groove on the surface of the enzyme that can accommodate at most six nucleotides [31]. However, as described above, these relative reaction rates reflect the specificity constants for the different substrates ( $k_{cat}/K_m$ ) and even if the  $k_{cat}$  values for the different substrates were similar,  $K_m$  is



only equal to the dissociation constant for the enzyme-substrate complex under limiting conditions where the rate of dissociation of the complex is fast relative to  $k_{\text{cat}}$ . Thus, it was of interest to use an independent method to compare directly the relative binding affinities of the enzyme for the different substrates. Figure 3 shows a series of gel-shift experiments for the same four substrates employed in the substrate competition analyses. To prevent cleavage of the substrates during the course of the assays, the catalytically inactive H263A mutant form of human TDP1 was used in these gel shift binding experiments [29]. Although a discrete shifted species could only be seen for the 19-pep and 28-pep substrates (Figure 3C and D), it appears that the affinity of the enzyme for the DNA-peptide complexes does indeed increase as the length of the DNA increases from 6 to 28. For the longer substrates, the substrate concentration at which half of the substrate was shifted to a slower mobility [42] yielded approximate  $K_d$  values of 130 nM and 40 nM, respectively, confirming the impression from the raw data that TDP1 does indeed have a higher affinity for 28-pep than for 19-pep. The value of 130 nM for the 19-pep compares very well with a previously-published value of 150 nM for a similar single-stranded substrate with a length of 16 nucleotides [19]. The apparent super-shifted species for 28-pep at the four highest substrate concentrations (Figure 3D) was possibly due to the binding of two molecules of TDP1 to this longer substrate. These results strongly suggest that the contribution of increasing DNA length to the binding affinity has a significant effect on the reaction rates observed in the substrate competition experiments. Thus, it appears that the proposed interaction of the DNA with the previously-observed short narrow channel in the crystal structure [31, 34] is insufficient to explain the effects of increasing DNA length on reaction rates.

### Effect of topoisomerase I peptide size on TDP1 cleavage

To generate a series of TDP1 substrates with increasing polypeptide lengths, we took advantage of the observation that combining certain pairs of topoisomerase I fragments reconstitutes near wild type enzyme activity [37, 38]. The schematic shown in Figure 4 illustrates how these reconstituted forms of the enzyme, when exposed to a suicide substrate, will produce topoisomerase I-DNA covalent complexes with the fragment containing the active site Y723 attached to a 12-mer DNA. The pairs of fragments used for the reconstitution experiments in this study are as follows: topo6.3/topo56, topo12/topo58, topo39/topo31 (Figure 5A). After suicide cleavage, subsequent purification of the DNA-protein complexes by SDS-PAGE followed by renaturation of the proteins (Material and Methods) generated the TDP1 substrates 12-topo6.3, 12-topo12, and 12-topo39. As a control substrate, we used the same suicide oligonucleotide to generate 12-topo70 (Figure 5A), which we have shown previously is a very poor substrate for TDP1 [24].

Each of the protein-DNA complexes was incubated with TDP1 for 20 min. Prior to the analysis of the TDP1 cleavage reactions by electrophoresis in a sequencing gel, the reactions were treated with trypsin to convert all of the remaining substrate to 12-pep which was well separated in the gel from the 3' phosphorylated 12-mer that is the product of the TDP1 reaction (Figs. 4 and 5B). Since a small amount of suicide cleavage occurred two nucleotides upstream of the major cleavage site (small arrow, Figure 4), some of the substrate contained a 10-mer oligonucleotide instead of the 12-mer, resulting in a 10-P product and a residual substrate band called 10-pep.

It has been shown previously that denaturation of the protein moiety of a protein-DNA complex renders the linkage between the protein and the DNA susceptible to TDP1 cleavage regardless of the size of the protein [14, 24]. Thus an important control for the cleavage experiments that follow below was to establish what fraction of the protein had properly folded during the renaturation step of the substrate preparation. To determine the efficiency of renaturation, we measured the ability of the protein in the purified complexes (12-topo6.3, 12-topo12, 12-topo39 and 12-topo70) to support the topoisomerase I religation

reaction. This measurement required the addition of the complementing protein fragment, a longer complementary oligonucleotide, and an acceptor oligonucleotide for the religation reaction as shown in Figure 4 (thick lines depict the added components). The fraction of the labeled oligonucleotide converted to the 25-mer religation product provides a measure of the amount of properly folded protein in each substrate preparation. Only for those substrates where the percentage of the protein-DNA complex that is cleaved by TDP1 exceeds the percentage that remains unreligated and therefore not properly folded, can we infer that the complex containing the native protein is a functional substrate for the enzyme.

The results of a representative experiment are shown in Figure 5(B). It can be seen that 12-topo12 and 12-topo6.3 are essentially completely cleaved to produce predominantly the 12-P product with a small amount of 10-P (lanes 6-9). Quantifying the radioactive bands revealed that the religation efficiency for 12-topo12 and 12-topo6.3 was only 23% and 21% respectively, but the corresponding percentages remaining unreligated (77% and 79%, respectively) was clearly less than the percent cleavage (Table 1). Thus both the 53 amino acid topo6.3 and the 108 amino acid topo12 are cleaved from the DNA in the native state. On the other hand, the percentage of 12-topo70 and 12-topo39 cleaved by TDP1 was less than the percentage remaining unreligated (Figure 5B, Table 1) and therefore all of the cleavage could be accounted for by the fraction of the topoisomerase I fragment remaining unfolded and unable to support the religation reaction. The small amount of cleavage observed here for the 12-topo70 substrate was comparable to that observed previously for a topo70-derived substrate which had never been subjected to a cycle of denaturation and renaturation [24], suggesting that the protein had effectively renatured under the conditions used here. It is unknown why the topo12 and topo6.3 proteins did not renature as well as the topo70 protein (Table 1). Taken together, these results indicate that the topoisomerase I-derived portion of a TDP1 substrate can be as long as 108 amino acids (topo12), but not as long as 333 amino acids (topo39) for efficient cleavage by TDP1.

### Dependence of TDP1 cleavage on *in vivo* proteolysis

The mutation in the *TDP1* gene that causes the neurological disease SCAN1 changes H493 to an arginine and results in the accumulation of the TDP1-DNA covalent reaction intermediate [17]. We have previously demonstrated that this covalent intermediate can be detected after exposure of SCAN1 mutant cells to CPT by a modified *in vivo* complex of enzyme (ICE) assay in which protein-DNA covalent complexes are separated from the bulk of the protein in cell extracts by sedimentation through a CsCl cushion with a density of 1.5 g/cc [18, 43]. We reasoned that if the activity of the proteasome were to be required prior to TDP1 cleavage as suggested by the *in vitro* data [24, 35], then the presence of a proteasome inhibitor before the CPT treatment should block the formation of the H493R TDP1-DNA covalent complexes in SCAN1 cells. Consistent with our earlier results [18], both topoisomerase I and TDP1 were detected by an immunoblot assay in the ICE pellet after CPT treatment of SCAN1 cells, but not in the control cells without the drug (Figure 6A, lanes 1 and 2). The presence of proteasome inhibitors MG132 or lactacystin in the cultures prior to the addition of CPT prevented the appearance of the H493R TDP1 in the protein-DNA complex fraction of the ICE assay (Figure 6A, lanes 4 and 6). The control immunoblots of samples from the whole cell extracts used in the ICE analyses (Figure 6B) showed that all of the extracts contained equal amounts of TDP1. These results demonstrate that proteolysis of the topoisomerase I moiety of the enzyme-DNA covalent complex is a prerequisite for TDP1 cleavage.

### Discussion

As a repair enzyme, TDP1 exhibits considerable versatility in removing a diverse spectrum of adducts from the 3' end of DNA. In addition to a tyrosine residue, the enzyme can



hydrolyze 3' phosphodiester linkages involving short peptides, a nucleoside, an abasic residue, an artificial biotin adduct, and a glycolate moiety [10, 22-24]. The feature common to all of these substrates is DNA, but there is a discrepancy between the biochemical and structural observations concerning whether the DNA is single or double stranded. In a crystal structure containing an oligonucleotide six bases in length, only the three nucleotides closest to the scissile phosphate are visible and they occupy a narrow groove extending away from the active site [31, 34]. This groove extends beyond the electron density of the DNA for approximately another three nucleotides, leading to the conjecture that the DNA portion of the substrate contacts the surface of the enzyme within this groove for a total of about six nucleotides. Importantly, modeling studies indicate that this groove is too narrow to accommodate duplex DNA [31, 34].

On the other hand, biochemical studies indicate that a substrate containing duplex DNA is as effectively cleaved as those with single-stranded DNA [13, 14, 19]. To address whether binding of a duplex substrate caused melting of the DNA near the site of cleavage, Raymond et al. [19] asked whether mismatches in the DNA would improve cleavage of a duplex substrate by favoring the melting process. They found instead that substrates with mismatches were poorer rather than better substrates. To reconcile the biochemical results with the crystal structure findings, they suggested an alternate model in which the first three nucleotides of a duplex DNA follow the path described for single-stranded DNA, but instead of continuing in the narrow groove, the DNA was proposed to bend upwards and track along one of the faces of the protein adjoining the groove [19]. While this model nicely accounts for the ability of the enzyme to cleave both single- and double-stranded DNAs with near equal efficiency, it fails to account for the DNA length dependence observed in the present study.

Our results suggest that the affinity of TDP1 for the DNA portion of the substrate increases as the length of the DNA is increased from 6 to 19 nucleotides and modestly increases further on extending the length to 28 nucleotides. Similarly, it was found by fluorescence anisotropy analysis that the affinity for a substrate with a 50-mer DNA was significantly greater than a very similar substrate containing a 10-mer DNA [20]. Although this effect could, in principle, be attributable to sequence rather than length, we regard this as unlikely since the  $K_m$  values determined by other investigators were nearly identical despite completely different DNA sequences used for the substrates [35, 44, 45].

To explain these observations on length dependence, we propose that the DNA, whether single- or double-stranded, follows the trajectory out of the active site proposed by Raymond et al. [19], and that intimate contact of the DNA with the surface of the enzyme is extended by a wrapping of the DNA around the protein. This suggestion is consistent with the observation that the protein surface predicted to contact the DNA is largely positively charged [31, 32]. Our data in combination with those of Raymond et al. [19] indicate that the trajectory of the DNA on the surface of TDP1 points away from the narrow groove originally suspected to be occupied by the DNA [32] and likely remains in contact with the surface of the protein for up to two turns of the DNA helix. If this suggestion is correct, it is likely that TDP1 can initially bind to DNA at internal sites before the 3' end is inserted in the active center of the enzyme as recently suggested by Dexheimer et al. [20]. Definitive proof for this hypothesis must await a co-crystal structure of TDP1 containing a bound DNA on the order of 20 nucleotides in length.

Previous results have shown that covalent complexes between DNA and the native form of a protein are refractory to cleavage by TDP1 [14, 24, 35]. The size of the protein alone is not the issue, since denaturation by heat or detergent renders the linkage susceptible to TDP1 [14, 24]. Interestingly, the covalent intermediate in the TDP1 reaction is susceptible to

cleavage by TDP1 itself without denaturation of the protein [24]. The structural basis for this curious exception is unknown, but it seems unlikely to be due simply to the fact that TDP1 is linked to the DNA through a phosphoamide bond involving histidine whereas the bond to a type IB topoisomerase is a tyrosine phosphodiester linkage. In both cases, the bond that must be cleaved by TDP1 is buried within the protein [32, 47] and the lack of accessibility is probably sufficient to explain why the native topoisomerase I linkage is not cleaved. One explanation for the susceptibility of the TDP1-DNA bond is that the interaction of this substrate with TDP1 sufficiently unfolds the protein to expose the scissile bond in the complex to the active site of the TDP1.

Our finding that a covalent complex involving a folded form of the C-terminal-most 53 amino acids of human topoisomerase I is susceptible to TDP1 cleavage is consistent with earlier structural models in which a C-terminal topoisomerase I fragment 46 amino acids in length was successfully docked into the protein binding pocket of TDP1 [32]. Here we show that the fully folded 108 amino acid topoisomerase I peptide can generate a complex that is still cleaved by TDP1. However, increasing the length of the peptide to 333 amino acids produces a substrate that, like the full length native topoisomerase I protein, is resistant to TDP1. These results show that, prior to cleavage by TDP1, the protein moiety of the topoisomerase I-DNA covalent complexes must either be unfolded or degraded by proteolysis to a length somewhere in the range of 108 to 333 amino acids.

A variety of studies indicate that the proteasome is involved in the degradation of topoisomerase I after treatment of cells with CPT [48-52], but these studies have not directly addressed whether this breakdown was required for the action of TDP1. However, a previous study demonstrated that MG132 blocks the conversion of CPT-induced topoisomerase I complexes in SCAN1 cells to single-strand breaks that can be detected by the alkaline comet assay [21]. Presumably the single-strand breaks that form in the absence of the proteasome inhibitor still retain a topoisomerase-related peptide that is not further repaired in the absence of TDP1 in SCAN1 cells, but is rapidly repaired in normal cells. Here we show by a more direct assay that in the presence of CPT, proteasome inhibitors block cleavage by the H493R mutant form of TDP1 and thereby prevent the trapping of the mutant enzyme on the DNA. Therefore the combined results of two independent studies using different methods strongly support the view that substrate processing by proteolysis is a prerequisite to cleavage by TDP1.

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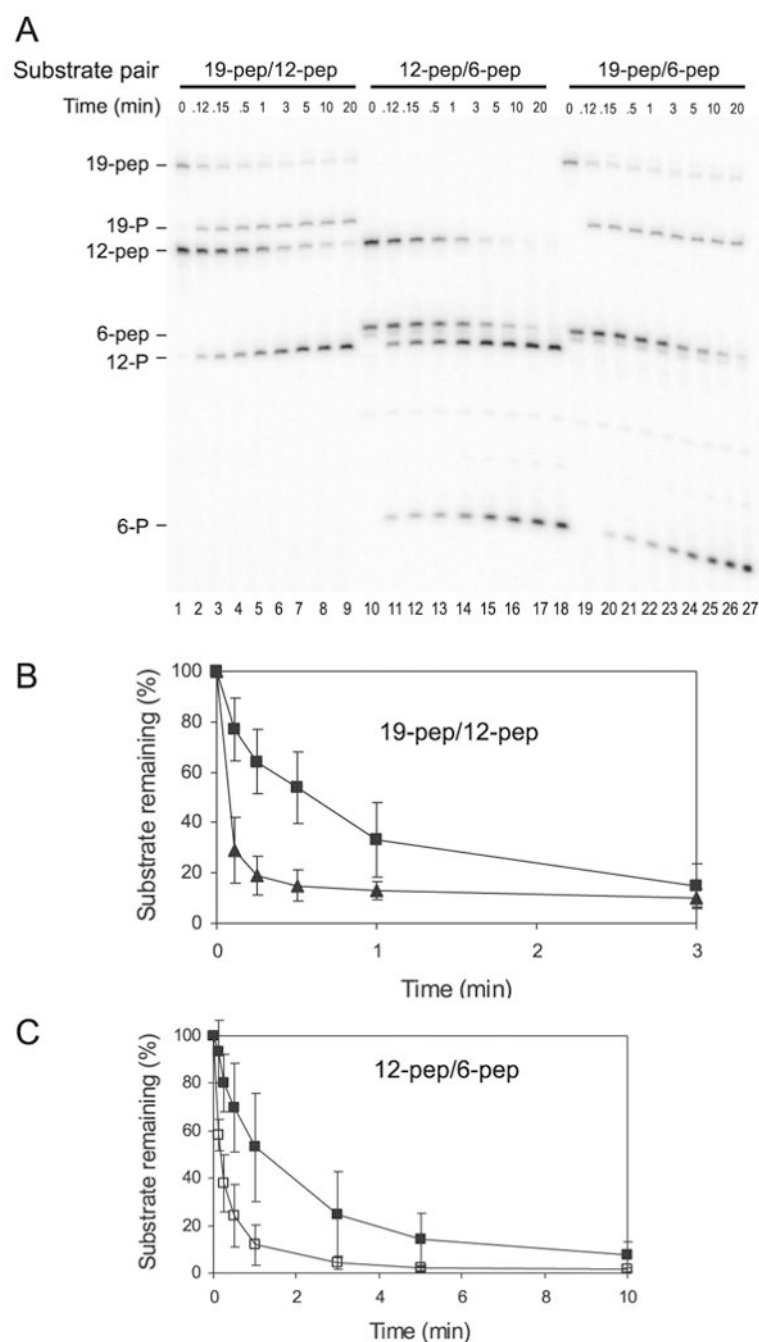
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## Abbreviations used

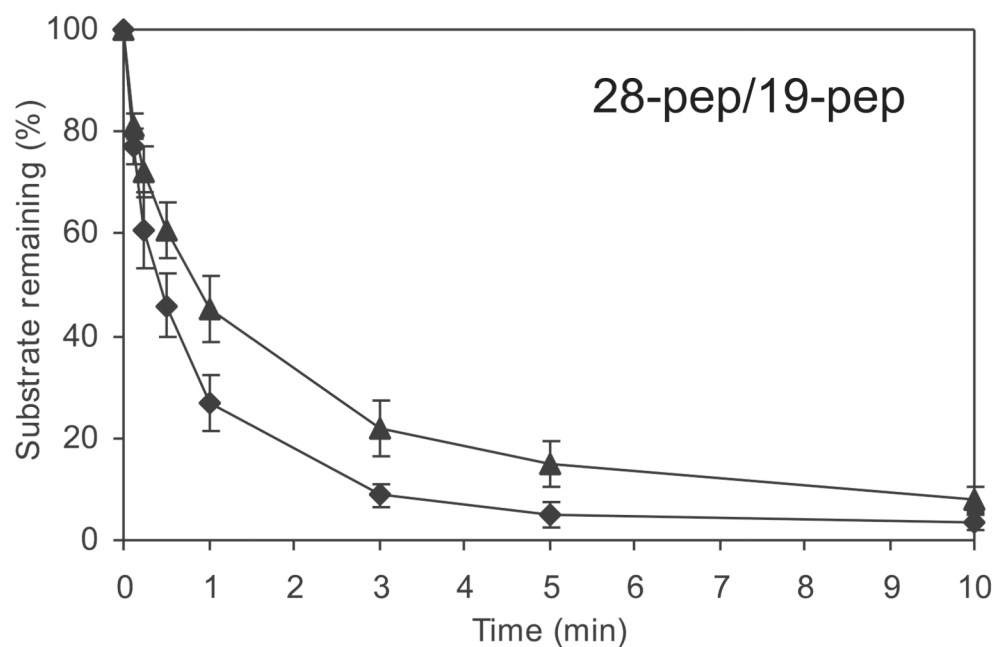
<b>CPT</b>	camptothecin
<b>TDP1</b>	Tyrosyl-DNA phosphodiesterase
<b>DTT</b>	dithiothreitol
<b>ICE</b>	in vivo complex of enzyme
<b>SCAN</b>	spinocerebellar ataxia with axonal neuropathy



**Figure 1. TDP1 substrate competition experiments**

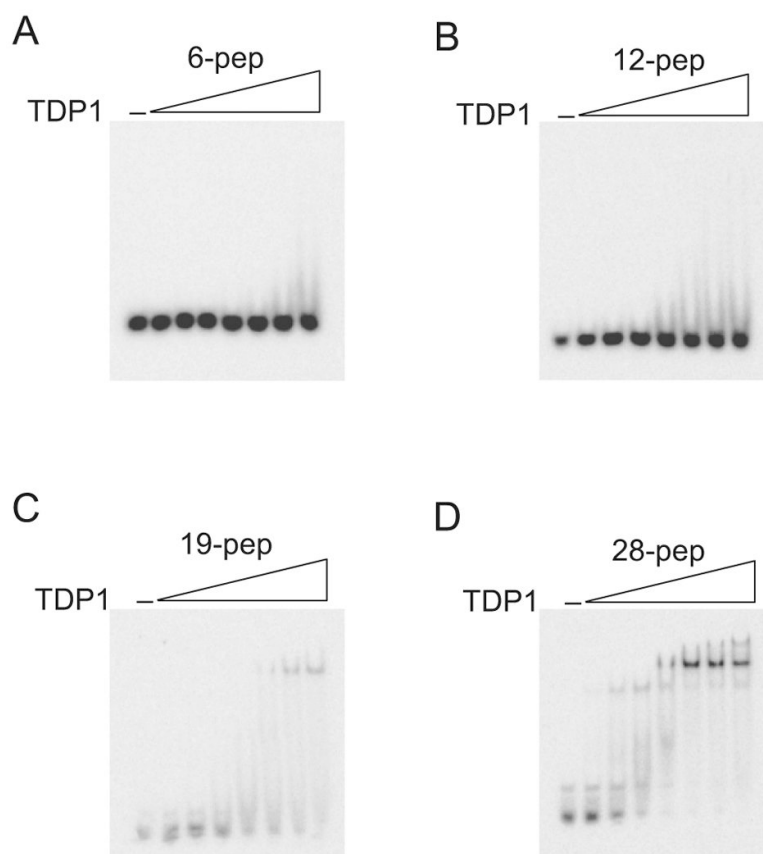
(A) Pairs of 5' end-labeled TDP1 substrates (19-pep/12-pep, 12-pep/6-pep, and 19-pep/6-pep) were incubated for the indicated times with TDP1 and the products analyzed by electrophoresis in a sequencing gel. The mobilities of the substrates and products (19-P, 12-P, and 6-P) are indicated along the left side of the panel. (B) and (C) For each experiment, the percentage of the substrate remaining at each time point was quantified by PhosphorImager analysis using the ImageQuant software and the results depicted graphically. Results are means  $\pm$  S.D. for three independent experiments. Panel B: closed triangles, 19-pep; closed squares, 12-pep. Panel C: open squares, 12-pep; closed squares, 6-pep.



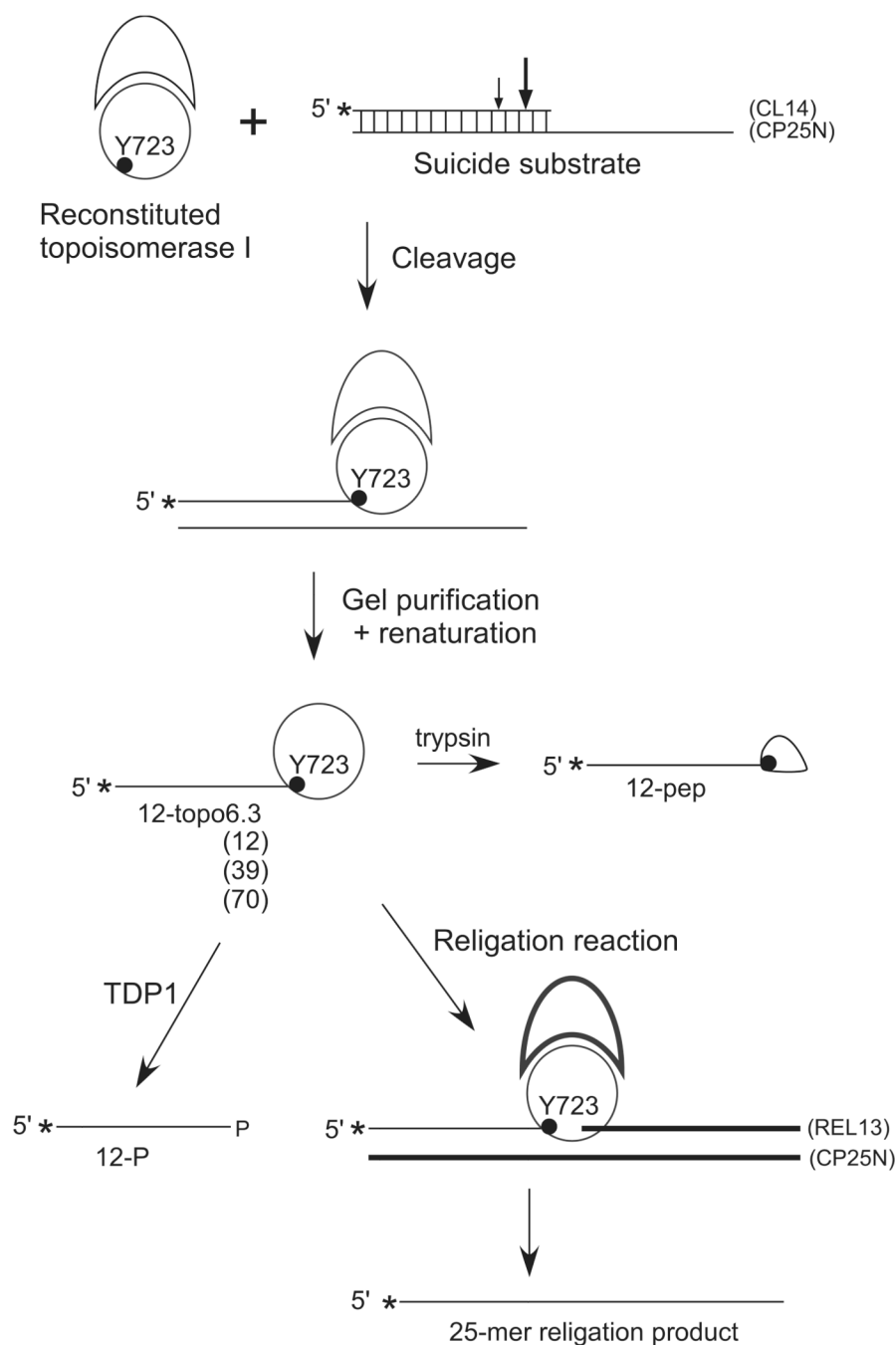


**Figure 2. TDP1 competition experiment using the 28-pep/19-pep pair of substrates**

The results for the 28-pep/19-pep competing pair of substrates are shown graphically as described for Figure 1. Results are means  $\pm$  S.D. for six independent experiments. Closed diamonds, 28-pep; closed triangles, 19-pep.

**Figure 3. Gel shift experiments**

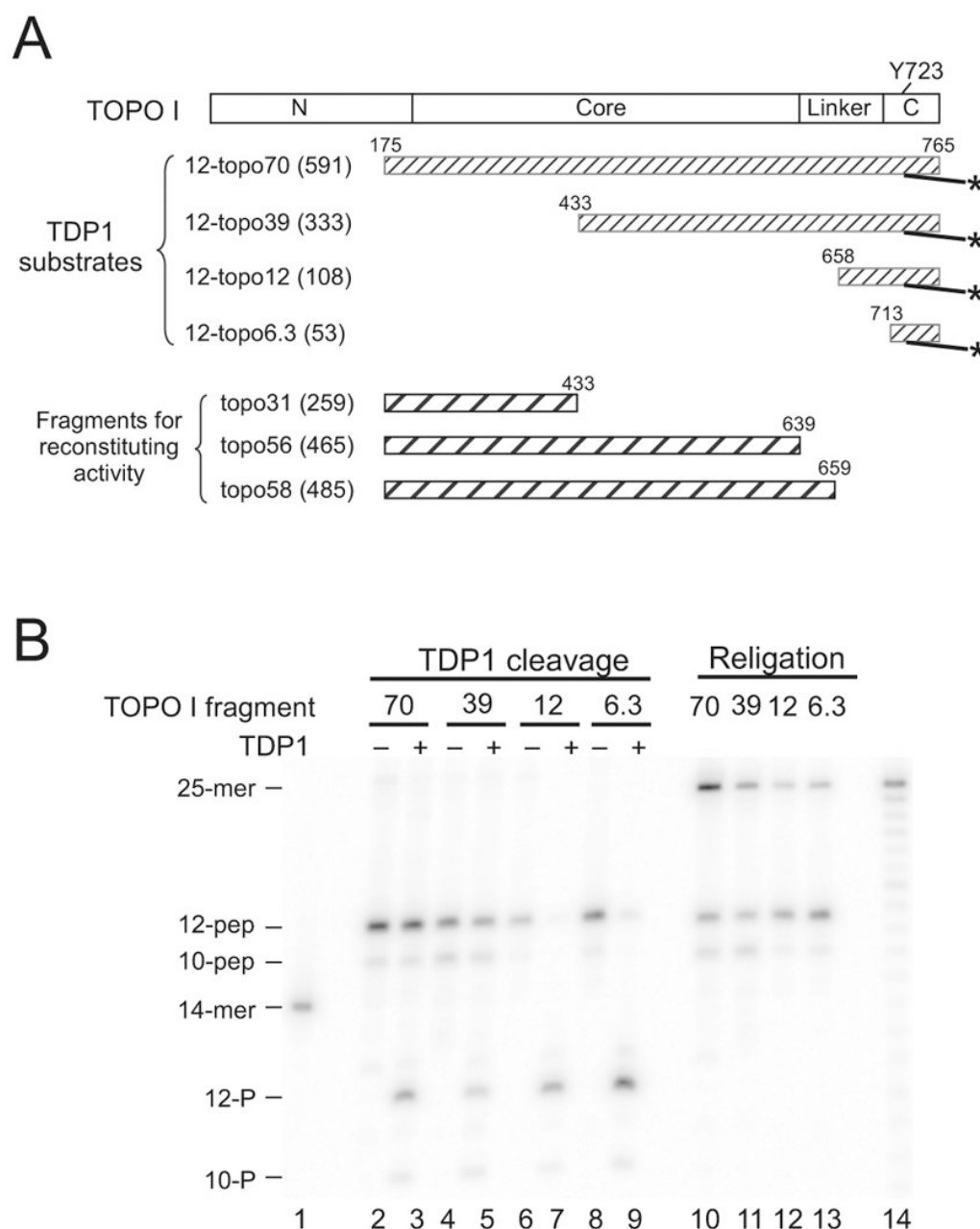
The indicated 5' end-labeled TDP1 substrates were incubated with two-fold increasing concentrations of TDP1 (from 23 nM up to 1.46  $\mu$ M) and subjected to native gel electrophoresis. The gels were dried and analyzed using the PhosphorImager (**A-D**). The first lane in each panel shows the mobility of the substrate in the absence of added TDP1.



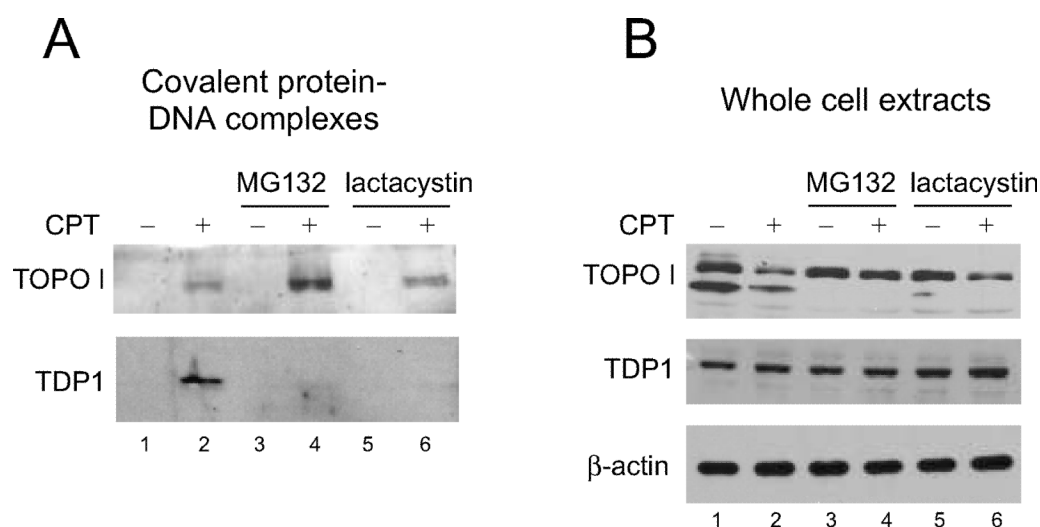
**Figure 4. Schematic showing the experimental protocol for varying the size of the protein portion of the TDP1 substrate**

A reconstituted form of human topoisomerase I was used to cleave a 5' end-labeled suicide substrate containing a 14-mer scissile strand and a 25-mer non-scissile strand. The major and minor suicide cleavage sites in the substrate are indicated by the large and small arrows, respectively. After cleavage, the C-terminal fragment containing the active site tyrosine (Y723) linked to DNA was gel-purified and subjected to renaturation conditions. This procedure was used to generate the following four TDP1 substrates: 12-topo6.3, 12-topo12, 12-topo39, 12-topo70. The product from treating these substrates with TDP1 was 5' end-labeled 12-P (minor cleavage site produced 10-P, not shown). Prior to the sequencing gel

analysis of the products, the reactions were treated with trypsin to convert all of the substrates into 12-pep (and a small amount of 10-pep, not shown). The extent refolding of the proteins was evaluated by the capacity of the renatured complexes to carry out the religation reaction to REL13 to produce a 25-mer religation product. The components that were added to the substrates for the religation reaction are indicated in the figure by the thick lines (lower right).



**Figure 5. Dependence of TDP1 cleavage on the size of the protein portion of the substrate**  
**(A)** The domain structure of human topoisomerase I is drawn to scale and shown across the top of the panel. The start and end points (indicated by amino acid position) of the TDP1 substrates and the reconstituting fragments are shown schematically below with the number of amino acids in the protein fragments indicated in parentheses. The line with the asterisk on the substrates denotes the 5' end-labeled 12-mer that is covalently attached to the active site tyrosine. **(B)** The TDP1 substrates were incubated without (-) and with (+) TDP1 followed by treatment with trypsin and analysis by sequencing gel electrophoresis (lanes 1-9). The mobilities of the substrates and products are given along the left side of the panel. The products of the religation reaction for each of the substrates are shown in lanes 10-13. The 14-mer and 25-mer marker DNAs were included in lanes 1 and 14, respectively.



**Figure 6. Effect of proteasome inhibitors on cleavage by H493R mutant form of TDP1**

(A) SCAN1 cells were incubated with CPT with and without prior treatment with either MG132 or lactacystin and the high molecular DNA was isolated by the ICE assay (lanes 2, 4, 6). Control incubations without CPT were included (lanes 1, 3, 5). The DNA pellets from the ICE assays were treated with Micrococcal nuclease and subjected to immunoblot analyses probing for topoisomerase I (topo) or TDP1. (B) Samples of the whole cell extracts used in the ICE assay were similarly subjected to an immunoblot analysis. In addition to probing for topoisomerase I and TDP1, the blots were probed for  $\beta$ -actin as a loading control.



**TABLE 11**

Substrate	Percent cleaved by TDP1	Percent unreligated
12-topo70	28 ± 3	36 ± 5
12-topo39	25 ± 2	52 ± 3
12-topo12	96 ± 2	77 ± 2
12-topo6.3	95 ± 2	79 ± 2

<sup>1</sup> Values are the average of two independent analyses of the samples shown in Figure 5B with the indicated errors reflecting the observed range.